

RESEARCH ARTICLE

Influence of rainfall and temperature on the spermatogenesis of *Leptodactylus macrosternum* (Anura: Leptodactylidae)

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ABSTRACT. In the semi-arid environment, the reproductive success of anurans depends on adaptations in their life cycle, which synchronizes with ideal environmental conditions to maximize the number of offspring. In this study changes in the histological and morphometric aspects of the testes of *Leptodactylus macrosternum* Miranda-Ribeiro, 1926 are characterized, to evaluate the influence of rainfall and temperature on them. Specimens were collected at Horto Florestal Olho d'Água da Bica – HFOB (06°49'20"S, 36°15'85"W) area, municipality of Cuité, state of Paraíba, Brazil. Search for specimens was active, happened at night, and amounted to 15 days from January to December 2013. The densities of spermatids (primary and secondary), sperm and area of the seminiferous locules of the testes were registered. The influence of climate variables (rainfall and temperature) on the density of primary and secondary spermatids, sperm and locular area were verified using Simple Linear Regression. Primary spermatids had the lowest density in July ($57.90 \pm 51.54 \text{ mm}^2$), with a peak in November ($300.32 \pm 117.35 \text{ mm}^2$); secondary spermatids had the lowest density in December ($287.87 \pm 79.05 \text{ mm}^2$), with a peak in May ($135,727.00 \pm 301.13 \text{ mm}^2$); sperm was in the lowest density in July ($237.37 \pm 121.10 \text{ mm}^2$), with a peak in June ($2,270.45 \pm 602.62 \text{ mm}^2$) and the locular area had the lowest density in December ($40,292.9 \pm 8,174.20 \mu\text{m}^2$) and highest density in June ($338,875.01 \pm 2,262.10 \mu\text{m}^2$). A notable decrease in sperm density was evident between June and July. That, associated with the observation of a larger locular area in June, allowed us to identify as June as the peak of spermatogenesis and the following month as the most potentially reproductive. The density of secondary spermatids ($r = 0.02$), sperm ($r = 0.21$) and locular area ($r = 0.01$) showed dependency on rainfall whereas only sperm ($r = 0.09$) showed dependency on temperature. Therefore, we can state that the reproductive cycle of *L. macrosternum* is potentially continuous, with a reproductive peak in July.

KEY WORDS. Anuran, semi-arid, spermatids, spermatozoa.

INTRODUCTION

In anurans, the control of spermatogenesis and consequently the reproductive cycles, are mediated by the interaction between the animal's endogenous rhythms and the influence of

environmental factors (Sasso-Cerri et al. 2004). Among the latter, temperature and rainfall are the main climatic factors influencing the reproductive cycles of anurans (Cardoso and Martins 1987).

The reproductive cycles of anurans are classified based on the sum of the histological features of their gonads and their

behavioral patterns as: continuous, potentially continuous and discontinuous (Lofts 1974). The discontinuous cycle, typical of temperate climate zones, is characterized by a discreet breeding period with pronounced changes in gonad size, gamete production and accessory sexual structures. Continuous and potentially continuous cycles, typical of tropical regions, are characterized by the constant production of gametes. However, in the latter, there is a partial interruption in gametogenesis during certain periods.

Typical of semi-arid regions, potentially continuous cycles are determined by irregular and limited rainfall (Duellman and Trueb 1994, Moore et al. 2005, Sasso-Cerri et al. 2004) and are thus limited to a short period during the year (Bertoluci and Rodrigues 2002, Rodrigues 2003, Prado and Uetanabaro 2000). In semi-arid regions, rainfall and temperature influence the maturation of the testes and consequent production of sperm (Sasso-Cerri et al. 2004), coinciding with the higher peaks of sex hormones, either for a short period (explosive reproduction) or permanently (prolonged reproduction) (Wilczynski et al. 2005). Rainfall and temperature also determine the timeline of the development of secondary sexual characteristics and the beginning and maintenance of sexual behaviors in anurans (Moore et al. 2005).

Leptodactylus macrosternum Miranda-Ribeiro, 1926 is widely distributed in semi-arid environments. It displays a generalist behavior and is well adapted to disturbed areas (De La Ivã and Maldonado 1999). The species is hunted for its meat (Alves et al. 2009) and is sporadically reared as an alternative to exotic species in local raniculture – e.g., *Lithobates catesbiana* (Shaw, 1802) (Cunha and Delariva 2009). Furthermore, due to its commercial value, abundance and easy detectability, *L. macrosternum* has been adopted as a biological model. Its biological characteristics make it an ideal subject to test our hypothesis that, in semi-arid regions, rainfall and temperature are the most important regulators of the spermatogenic process in Anuran Amphibians. In this study we characterize the histologic and morphometric aspects of the testes of *L. macrosternum* and evaluate the influence of rainfall and temperature on the parameters tested.

MATERIAL AND METHODS

Night expeditions were undertaken every 15 days, from January to December 2013, to verify the reproductive activity of *L. macrosternum* males. According to Arzabe (1999), whose work was carried out in the semi-arid environments of the state of Paraíba, anurans subjected to extreme hydric stress present a short and explosive reproductive period. Therefore, to increase our chances of capturing specimens in different stages of spermatogenesis, our collecting expeditions were 15 days apart from one another.

Males of *L. macrosternum* were collected manually at Horto Florestal Olhod'Água da Bica-HFOB (06°49'20"S, 36°15'85"W), located in the municipality of Cuité, Paraíba. The area is situated in the Brazilian Caatinga domain and is characterized by its hot,

dry climate with temperatures ranging between 17 and 28 °C, an average monthly rainfall of 76.35 mm and relative humidity of approximately 70%. The hydrography of the Caatinga is peculiar, with temporary rivers, ponds, streams, reservoirs, wetlands and as slope areas from which the sources of water flow. The Horto Florestal encompasses 70 ha of bush and arboreal caatinga (Abrantes et al. 2011).

The monthly weather temperature (°C) and rainfall (mm) data were obtained from the Agência Executiva de Águas do Estado da Paraíba (AESA 2014) database, at the weather station neighboring the sampling location.

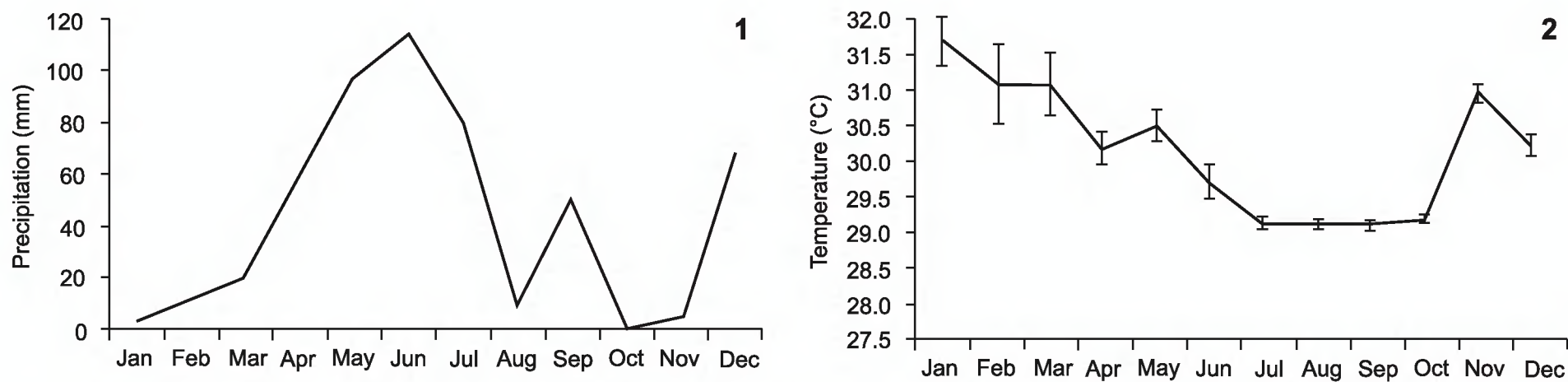
The captured males of *L. macrosternum* were taken to the Biosystematics Amphibian Laboratory (LABAN) of the Universidade Federal de Campina Grande, Cuité Campus, Paraíba, where they were euthanized with an overdose of lidocaine 5%.

The testes were fixed in Bouin solution (a mixture of 71% picric acid, 24% pure formaldehyde and 5% of glacial acetic acid) for 24 hours, then dehydrated in increasing alcohol series (70%-30 min, 80%-30 min, 90%-30 min, 95%-30 min, 100%-30 min and 100%-30 min) (Hopwood 1990), and later paraffin embedded, sectioned at 5.0 µm and stained with hematoxylin and eosin (Robinson and Gray 1990). Qualitative analysis of the slides was conducted in a conventional, trinocular, bench microscope (Olympus AX70, Tokyo, Japan – Department of Morphology, Universidade Federal do Espírito Santo – UFES), coupled to a digital image acquisition system (camera ERc 5s and Axiovision 6.3, Carl Zeiss, Jena, Germany from UFES). The cell types were identified according to work described by Hermosilla et al (1986 and Oliveira et al (2003a, b). For the analysis of the seminiferous locular area, a total of twenty-seven loci were randomly selected (raffled), with three animals being analyzed per month (nine loci each); a total of 162 area measurements (Santos and Oliveira 2007).

Stereological analysis to determine the density of spermatids and spermatozoa was based on the precepts of Mandarim-de-Lacerda (1995 and Weibel (1979). The volume density (V_v) of spermatids and spermatozoa were calculated as they represent excellent cellular models as bioindicators of the sexual maturation stage of the individuals. The sample calculation was performed with the use of the Hally (1964) formula and appropriately corrected to the result representing the stereological principles (Mandarim-de-Lacerda 1995). Lastly, to measure density profiles (Q_A primary and secondary spermatids and spermatozoa), 14, 5 and 6 fields were respectively numbered in a Test Area (TA) with 0.044 mm² for each animal per study group. The final result (mm²) was obtained using the mean for each profile as well as the following equation: $Q_A = \sum \text{profiles} / \text{TA}$.

The values for the density of cells in the reproductive lineage and the area of the seminiferous locules were compared between months using the Kruskal-Wallis test and, when necessary, supplemented by the Dunn test.

The dependency between the climate variables (rainfall and temperature), the density of the population of reproductive lineage cells, and the area of the seminiferous locules were



Figures 1–2. Climatological variations in the Horto Florestal Olho d'Água da Bica (HFODB) in the municipality of Cuité, state of Paraíba, Brazil between January and December 2013: (1) total rainfall (mm); (2) mean and standard deviation of the temperature (°C).

verified using a Simple Linear Regression test. All analyzes were based on Zar (1999) and $p < 0.05$ considered as a reference to assign statistical significance.

RESULTS

The highest rainfall rates were concentrated from April to July 2013. The lowest rainfall levels at the region were recorded in October and November (Figs 1, 2). As for the temperature variation during the sample period, the highest averages were recorded during January, March and November 2013 (Fig. 4).

Twenty-nine *L. macrosternum* males were collected in May (5 samples), June (11 samples), July (3 samples), August (4 samples), November (4 samples) and December (2 samples) 2013, coinciding with rainfall in the region. Between January and April, and September and October 2013, no individuals of *L. macrosternum* were found during field trips.

All cell types in the testes of *L. macrosternum* collected during the sample period were easily verified, with each locule featuring multiple cysts of spermatogenic cells (Fig. 3). Each cyst appeared as a group of germ cells in the same state of differentiation. By delimiting the locules, it was possible to observe the tunica albuginea (Fig. 3, TA), and the mediastinal region at the center of the testicular structure, (Fig. 3, Med). Both structures are formed by connective tissue.

Primary spermatogonia (Fig. 5, SG1), were recorded as cells located in the locular periphery, bordering the tunica albuginea and presenting granular chromatin. Secondary spermatogonia (Fig. 5, SG2) presented a more intense color in comparison with the other cysts, with cells situated immediately after the primary spermatogonia, directing towards the lumen of the seminiferous locule. Both cell types were similar in size.

Primary spermatocytes (Fig. 4, Sp1) were presented as large cells, although smaller than the primary spermatogonia, and had looser chromatin. Secondary spermatocytes (Fig. 5, Sp2) were slightly smaller than the aforementioned cells, with a denser nucleus and large eosinophilic cytoplasm area around it.

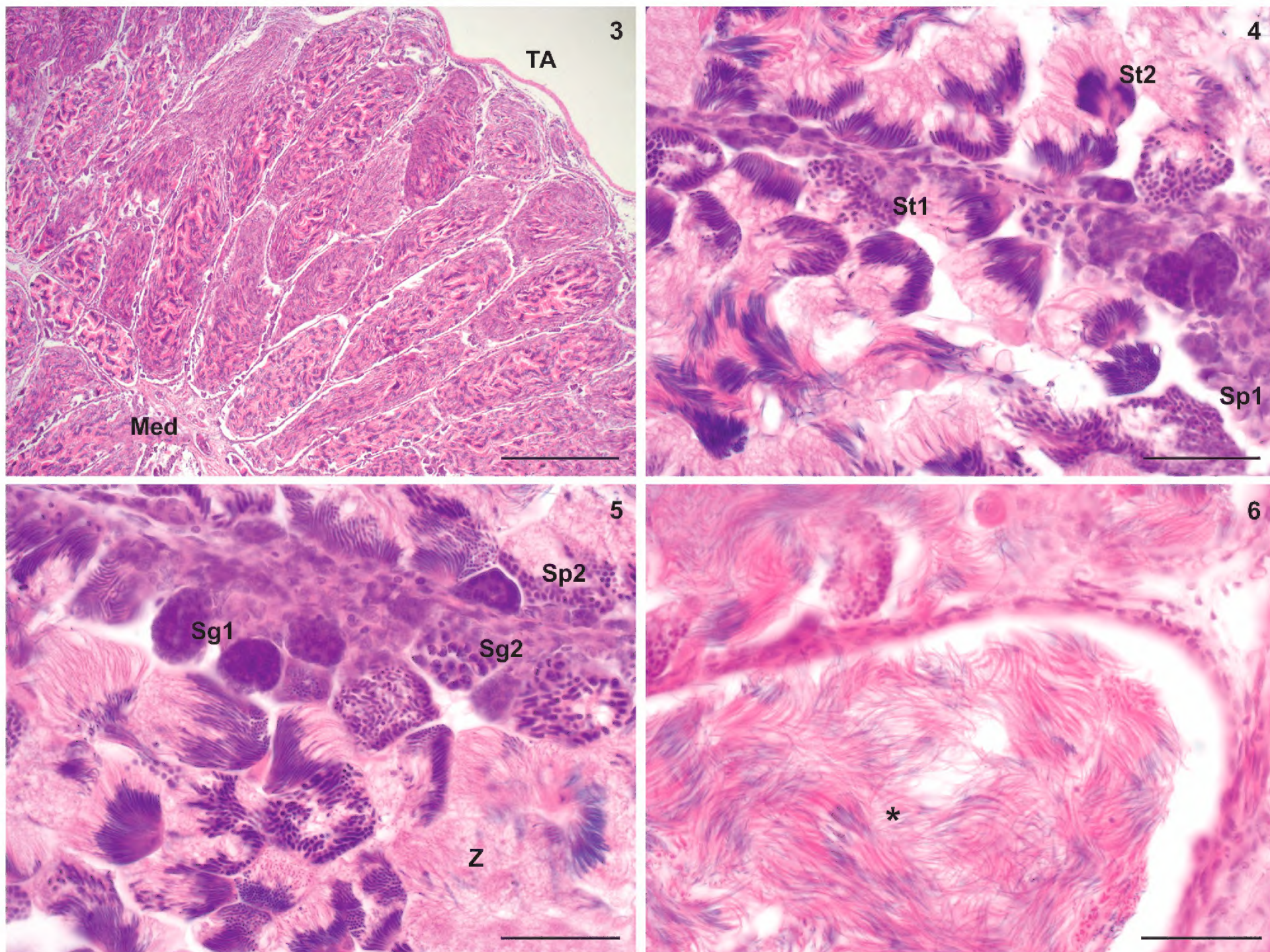
Table 1. Monthly values (mean and standard error) for density of primary spermatids (St1), secondary spermatids (St2), sperm (Ez) (mm^2) and locular area (μm^2) of the testes of *Leptodactylus macrosternum* species collected in the HFOB area, municipality of Cuité, state of Paraíba, Brazil, in the period from May to December 2013. * Significant differences ($p < 0.05$, Kruskal-Wallis).

	St1	St2	Ez	Locular Area
May	176.29 \pm 46.64	1357.27 \pm 30113*	1121.97 \pm 189.2*	227688.7 \pm 1274.1
Jun	93.18 \pm 58.35	1301.81 \pm 117.64*	2270.45 \pm 602.62*	338875.01 \pm 226.1*
Jul	57.90 \pm 51.54*	633.33 \pm 619.74	237.37 \pm 121.1*	96968.4 \pm 464.2*
Aug	83.19 \pm 37.12	770.45 \pm 298.04	1007.57 \pm 370.52	206367.2 \pm 719.3
Nov	300.32 \pm 117.35*	628.4 \pm 3090.36	602.27 \pm 212.83	281801.9 \pm 4736.6
Dec	139.06 \pm 26.22	287.87 \pm 79.05*	356.06 \pm 179.46	40292.9 \pm 8174.2*

Table 2. Relations between the density of primary spermatocytes (St1), secondary spermatocytes (St2), sperm (Ez) and the area occupied by the seminiferous locules (LA) in male *Leptodactylus macrosternum* species in relation to rainfall and temperature in the Horto Florestal Olhod'Água da Bica (HFOB) area in the municipality of Cuité, state of Paraíba, Brazil, in the periods from May to August and November to December 2013. * = Represents significant differences.

	Rainfall		Temperature	
	r	p	r	p
St1	0.03	0.40	0.01	0.77
St2	0.02	0.04*	0.05	0.20
Ez	0.21	0.04*	0.09	0.03*
AL	0.01	0.04*	0.01	0.75

Primary spermatids (Fig. 4, St1) were rounded cells, with a slightly compressed nucleus and somewhat elongated shape. Secondary spermatids (Fig. 4, St2) were cells in which the nucleus undergoes stretching concurrently with a progressive nuclear compaction, crumbling its cystic organization to arrange itself in bundles supported by Sertoli cells, although still being considered as a germinal cyst. The spermatozoa (Figs 5–6, Z)



Figures 3–6. Histologic sections of *Leptodactylus macrosternum* testes, stained in HE, collected between the months of May to August and November to December in the Horto Florestal Olhod'Água da Bica (HFOB) area in the municipality of Cuité, state of Paraíba, Brazil. (3) section at 40x: (TA) tunica albuginea, (Med) mediastinum; (4) section at 40x TA: (St1) primary spermatid, (St2) secondary spermatid, (Sp1) primary spermatocyte; (5) section at 40x TA: (Sg1) primary spermatogonia, (Sg2) secondary spermatogonia, (Sp2) secondary spermatocyte, (Z) sperm; (6) section at 40x: (*) lumen with sperm. Scale bars: 3 = 500 μ m, 4–6 = 50 μ m.

appeared as cells characterized by a nuclear compaction and a cytoplasmic reduction, often free in the center of the locules.

The monthly density values of the primary spermatids (St1), secondary spermatids (St2), sperm (Ez) and variation of the locular area of seminiferous tubules showed significant differences ($p < 0.05$, Kruskal-Wallis) over the months of collection (Table 1). The primary spermatid densities (St1) were significantly higher in November ($p = 0.01$), presenting the lowest significant values in July ($p = 0.03$). The highest density values for secondary spermatids (St2) were significantly higher in the months of May ($p = 0.01$) and June ($p = 0.02$), whereas the month of December ($p = 0.03$) showed important lower density values for secondary spermatids (St2). The densities of spermatozoa (Ez) also had the highest significant values in the

months of May ($p = 0.04$) and June ($p = 0.04$), with meaningful lower values in July ($p = 0.02$). Regarding the variation of the locular area of the testes of the animals analyzed, those sampled in the month of June showed the highest values ($p = 0.01$). The months of July ($p = 0.03$) and December ($p = 0.04$) presented important lower values for this parameter (Table 1).

As shown in Table 2, significant relationships were only observed between rainfall and the density of secondary spermatocytes (St2) ($R^2 = 0.02$, $p = 0.04$), sperm density (Ez) ($r = 0.21$, $p = 0.04$) and the area occupied by the locules in the seminiferous tubules (AL) ($r = 0.01$, $p = 0.04$) of the testes. The monthly temperature variation in the HFOB area only showed significant relationship with the density of sperm (Ez) ($r = 0.09$, $p = 0.03$).

DISCUSSION

Our results corroborate the results of Santos and Oliveira (2008), and Leite et al. (2015), who pointed out that, in general, the spermatogenesis process in anurans is cystic, with the organization of germ cells always in the same stage of differentiation. However, the organization of these cysts may vary among different families of anurans (proportion of germ cells present and/or absent) and especially according to the environment where these animals occur, as found by Ferreira et al. (2009) for *Dendropsophus nanus* (Boulenger, 1889), *Pseudis limellum* (Cope, 1862) and *Pseudis paradoxa* (Linnaeus, 1758) of Hylidae and the *Leptodactylus chaquensis* Cei, 1950 and *Leptodactylus podicipinus* (Cope, 1862), species of Leptodactylidae from the Pantanal region.

In our findings, the cell characteristics of *L. Macrosternum*, and the process of spermatogenesis, with variation in the quantities of cysts and especially the spermatozoa within the locules, are similar to *L. podicipinus* (Prado et al. 2002), *L. chaquensis* and *L. podicipinus* (Ferreira et al. 2009), *Leptodactylus fuscus* (Schneider, 1799) (Maragno and Cechin 2009) and *L. chaquensis* (Villagra et al. 2014), demonstrating an organizational pattern of germ cells dependent on the seasonal variation of each region.

This morphology, added to a constant production and partial interruption when conditions are unfavorable for reproduction, features an acyclic spermatogenesis process. This appears to be a characteristic of anurans from the *Fuscus* group of Leptodactylidae (Ferreira et al. 2009), without the observation of all stages of cell differentiation, but with the constant presence of elongated spermatids in a fan-shaped array (Fig. 4) and free spermatozoa (Fig. 6).

Therefore, the characteristics observed in the gametogenic process of *L. macrosternum* classify this type of reproductive cycle as “potentially continuous”. This type is typical of seasonal, tropical environments (Lofts 1974). Madelaire and Gomes (2016), studying *Rhinella granulose* (Spix, 1824), *R. jimi* (Stevaux, 2002) and *Pleurodema diplolister* (Peters, 1870) reported that, during the reproductive period in caatinga areas in the state of Rio Grande do Norte, Brazil, male anuran testes presented an advanced stage of maturation, with large germ cysts and beams of free sperm in the cysts’ lumen, corroborating the results presented here (Fig. 6).

With regards to the successive histological changes of the analyzed testes, the monthly density values of the primary spermatids (St1), secondary spermatids (St2), sperm (Ez) and variation of locular area of the seminiferous tubules varied significantly between the months of collection, besides portraying a chronological sequence that are compatible with the evolutionary stages of spermatogenesis (Table 1).

In tropical regions of seasonal climate, the reproductive activity of anurans is associated with the rainy season (Santos and Oliveira 2008). This is consistent with the lack of records for the dry period, between January and April, when humidity

is very low and rainfall rates fall in the Curimatau macroregion of the state of Paraíba.

In this study, the density of the secondary spermatocytes (St2), the density of sperm (Ez) and the monthly variation in the locular area of the seminiferous tubules (LA) (Table 2) of the testes of the individuals of *L. macrosternum* were significantly correlated with the rates of rainfall in the HFOB area. This corroborates the results of Prado et al. (2002), Prado and Haddad (2003) and Ferreira et al. (2009), who claimed that the reproductive period of Leptodactylidae anurans coincides with the period of higher rainfall in their occurrence areas. This fact may indicate that rainfall in caatinga areas is responsible for stimulating spermatogenesis in anurans of semi-arid environments (Madelaire and Gomes 2016), determining several physiological adaptations, especially those related to basal metabolism, especially to the reproductive cycle (Giaretta et al. 2008).

The monthly temperature variation in the HFOB area only showed significant relationships with sperm density (Ez) (Table 2) and no significant relationships with the remaining parameters. Thus, it is clear that, in semi-arid areas, the physical condition of the environment that interferes the most with the reproductive activity of anurans is rainfall, followed by temperature, determining the most favorable time for reproduction and consequently the spatial distribution of anuran reproduction in semi-arid regions (Duellman and Trueb 1994, Sasso-Cerri et al. 2004, Moore et al. 2005). However, the spermiogenesis and/or spermiation may be correlated with the temperature through the effect of high temperatures on testosterone levels (Kalt 1976, Rastogi et al. 1976). It is known that spermiogenesis lasts no more than 15-45 days, therefore (Rastogi et al. 1976) the effect of and the relationship with temperature on Ez density may be associated with this fact.

Our findings confirm the studies of Prado Uetanabaro (2000), Bertoluci and Rodrigues (2002), and Rodrigues (2003), and allow us to conclude that in environments where seasonality is significant, animals are reproductively active for two or three months during the year, depending on the rainfall peak, and consequently on the availability of reproductive sites for amplexus and the posterior development of tadpoles.

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